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Susceptibility testing of *Mycobacterium tuberculosis*: comparison of the BACTEC TB-460 method and flow cytometric assay with the proportion method

D. J. DeCoster[1,2], *R. M. Vena*[1,2], *S. M. Callister*[3] *and R. F. Schell*[1,2,4]

[1]Wisconsin State Laboratory of Hygiene, [2]Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, [3]Microbiology Research Laboratory, Gundersen Medical Foundation, La Crosse and [4]Department of Bacteriology, University of Wisconsin, Madison, WI, USA

ABSTRACT

Tuberculosis is a leading cause of morbidity and mortality worldwide. Susceptibility testing of the causative agent, *Mycobacterium tuberculosis*, is critical for control of the disease. This study compared the flow cytometric susceptibility assay with the proportion method and the BACTEC TB-460 system. There was agreement between the flow cytometric and proportion methods for 73 (94%) of 78 isoniazid tests, and complete agreement for 26 ethambutol and rifampicin tests. In contrast, the proportion and BACTEC methods failed to agree for 22%, 15% and 8% of isoniazid, ethambutol and rifampicin tests, respectively. These findings indicated that susceptibility testing by the flow cytometric assay is accurate, with results available within 24 h of initiation of the testing procedure.

Keywords BACTEC TB-460, flow cytometric assay, *Mycobacterium tuberculosis*, proportion method, susceptibility testing, tuberculosis

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INTRODUCTION

The USA Centers for Disease Control and Prevention consider that susceptibility testing of *Mycobacterium tuberculosis* is critical for treatment of tuberculosis patients and control of the disease [1]. Unfortunately, susceptibility testing of *M. tuberculosis* is limited by the time required to obtain results, primarily because of the slow growth of the organism. Conventional methods of anti-mycobacterial susceptibility testing, such as the proportion method, require incubation for *c*. 3 weeks before results are available [2–4]. Even the method used most frequently, BACTEC-460, requires incubation for 4–12 days [4–8]. Therefore, newer methods are being developed constantly to facilitate more rapid availability of susceptibility results [9–18].

Corresponding author and reprint requests: R. F. Schell, Wisconsin State Laboratory of Hygiene, 465 Henry Mall, Madison, WI 53706, USA
E-mail: rfschell@facstaff.wisc.edu

Previous studies have reported that susceptibility testing of *M. tuberculosis* could be accomplished rapidly by using a flow cytometer [18–20]. The method is based on the ability of viable *M. tuberculosis* cells to accumulate 5-chloromethylfluorescein diacetate (FDA) and hydrolyse the compound rapidly to free fluorescein by intrinsic cellular esterases [21–23]. The fluorescein accumulates in viable cells, while dead cells, or mycobacterial cells inhibited by anti-mycobacterial agents, hydrolyse significantly less FDA. The differences in fluorescein concentrations within viable or killed and inhibited mycobacteria can be distinguished easily by flow cytometric analysis. Multiplication of *M. tuberculosis* is not required, and reproducible results are available within 24 h [18,19].

In the present study, evidence is provided that the flow cytometric susceptibility assay is valuable for performing routine susceptibility testing of *M. tuberculosis*. The results of anti-tuberculosis susceptibility tests obtained by the proportion, BACTEC-460 and flow cytometric methods were

compared using *M. tuberculosis* isolates with varied susceptibility patterns to ethambutol, isoniazid and rifampicin.

MATERIALS AND METHODS

Anti-mycobacterial agents

Ethambutol, isoniazid and rifampicin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions (10 mg/mL) of ethambutol and isoniazid were prepared in distilled water, sterilised by filtration through a 0.2-μm filter and stored at −70°C in 1.0-mL aliquots until used. Rifampicin (10 mg/mL) was prepared similarly, except that it was prepared in dimethylsulphoxide (Sigma).

Mycobacteria and preparation

Twenty-six clinical isolates of *M. tuberculosis* with varied resistance to ethambutol, isoniazid and rifampicin were obtained from the Centers for Disease Control and Prevention and the Wisconsin State Laboratory of Hygiene. Reference *M. tuberculosis* strains ATCC 27294, 35822, 35838 and 35837 were also included in the study. Each isolate was grown from frozen stock in 10 mL of 7H9 broth (Wisconsin State Laboratory, WI, USA) in a sterile 10-mL glass screw-cap tube at 37°C in the presence of CO_2 5% v/v until the turbidity of the suspension was equivalent to a McFarland 1.0 standard (*c.* 5–14 days).

Agar proportion susceptibility testing

An agar proportion method, similar to that recommended by the NCCLS [2,4], was used to determine the percentage of *M. tuberculosis* cells resistant to each of the concentrations of anti-mycobacterial agents tested. Briefly, ethambutol, isoniazid and rifampicin were added to 7H10 medium, and held at 50–52°C, to yield final concentrations of ethambutol 5 mg/L, isoniazid 0.2, 1 and 5 mg/L, and rifampicin 1 mg/L. Subsequently, 5 mL of medium containing each anti-mycobacterial agent was dispensed into labelled quadrants of sterile Petri plates. One quadrant was reserved for 7H10 medium without any anti-tuberculosis agent. After solidification, the plates were inoculated with 0.1 mL of 10^{-2} and 10^{-4} dilutions of a McFarland 1.0 suspension of each isolate of *M. tuberculosis*, and then incubated at 37°C in CO_2 5% v/v for 3 weeks. An isolate was considered susceptible to an anti-mycobacterial agent if the number of colonies that grew on the drug-containing plate was <1% of the number of colonies that grew on the drug-free control. An isolate was considered resistant if ≥1% grew on the drug-containing plate.

Radiometric broth method (BACTEC)

Initially, 7H9 broths were inoculated (1 mL) with an isolate of *M. tuberculosis* and incubated (5–14 days) until turbidity matched a McFarland 1.0 standard (3×10^8 CFU/mL); this was followed by inoculation (0.1 mL) of a BACTEC 12B vial (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA). After incubation for 2–7 days at 37°C, aliquots (0.1 mL) of each working suspension with a growth index (GI) of 999 detected with the BACTEC 460 instrument (Becton Dickinson) were diluted 1:10 and 1:100 with BACTEC diluent. Subsequently, 0.1 mL of each of the diluted suspensions was used to inoculate drug-containing BACTEC 12B vials (ethambutol 2.5 mg/L; isoniazid 0.1, 0.4 and 2.5 mg/L; rifampicin 2 mg/L), together with a drug-free control. The vials were incubated at 37°C and tested in the BACTEC 460 instrument at approximately the same time each day. The MIC was interpretable when the GI of the 1:100 control read 20 or more for three consecutive days while the GI of the undiluted control read 999. It was necessary for these requirements to be met between days 4 and 8 of incubation for the test to be considered valid. The MIC was the lowest concentration of the anti-mycobacterial agent that inhibited 99% of the bacterial population. The final GI was <50.

Flow cytometric susceptibility test

An aliquot (0.9 mL) of each actively growing *M. tuberculosis* isolate was transferred to a 2-mL screw-cap microtube (Sarstedt, Newton, NC, USA). Solutions of isoniazid 50, 10 or 2 mg/L, ethambutol 50 mg/L or rifampicin 10 mg/L were then added (0.1 mL) to each tube. In other experiments, tubes containing 0.9 mL of mycobacterial suspension were supplemented (0.1 mL) with isoniazid 50, 25, 10, 5, 2, 1.2 and 0.6 mg/L. Drug-free suspensions of *M. tuberculosis* were included as controls. The suspensions were incubated for 24 h at 37°C in the presence of CO_2 5% v/v. After incubation, 0.2 mL of each suspension was removed and placed in a sterile 2-mL screw-cap microtube containing 0.2 mL of FDA (Molecular Probes, Eugene, OR, USA), prepared fresh on a daily basis at 500 ng/mL in phosphate-buffered saline, pH 7.4. The samples were then incubated at 37°C for 30 min before being analysed with a Bryte HS flow cytometer with WinBryte software (Bio-Rad Laboratories, Hercules, CA, USA).

Initially, unstained viable *M. tuberculosis* cells were detected and differentiated from non-*M. tuberculosis* particles in 7H9 medium by forward and side angle light scatter. Background events (particles) in the 7H9 medium and electronic noise were eliminated by thresholding. Subsequently, viable *M. tuberculosis* cells, incubated in the presence or absence of anti-mycobacterial agents for 24 h, were stained with FDA. For each isolate, at each concentration of anti-mycobacterial agent, the flow cytometer provided a histogram relating the number of *M. tuberculosis* cells in each of 2048 logarithmic channels of increasing fluorescence intensity, a mean channel fluorescence value, and a contour plot relating forward angle light scatter and intensity of fluorescence. In total, 2–10 000 events were acquired for each sample. In addition, fluorescent polystyrene beads with a diameter of 1.5 μm (Bio-Rad) were used daily for calibration of the instrument.

Flow cytometric susceptibility index

The susceptibility index was determined from the mean channel fluorescence value obtained from histogram profiles (channels 0–2048) of the population of FDA-stained *M. tuberculosis* cells in the presence or absence of anti-mycobacterial agents [18]. Subsequently, these values were divided by 512, i.e., the number of channels per log decade. The antilog was then determined for these values to obtain the relative linear fluorescence value for each sample analysed. Finally, the relative fluorescence value of each drug-containing sample was divided by the relative fluorescence value of the drug-free control to obtain the susceptibility index for each isolate. An isolate of *M. tuberculosis*

was considered susceptible to an anti-mycobacterial agent if the susceptibility index was ≤0.75. This value was set before experimentation. The calculation eliminated any variability among the isolates of *M. tuberculosis* in their ability to hydrolyse FDA in the absence of anti-mycobacterial agents.

Data reporting

The isoniazid concentrations used in the BACTEC method (0.1, 0.4 and 2.5 mg/L) were equivalent to concentrations of 0.2, 1 and 5 mg/L used in the proportion [4] and flow cytometric methods. Likewise, ethambutol 2.5 mg/L and rifampicin 2 mg/L were used in the BACTEC method, while ethambutol 5 mg/L and rifampicin 1 mg/L were assayed in the proportion [4] and flow cytometric methods. To simplify reporting of data, BACTEC concentrations are listed as isoniazid 0.2, 1 and 5 mg/L, ethambutol 5 mg/L, and rifampicin 1 mg/L.

RESULTS

Susceptibility of clinical isolates of *M. tuberculosis* to anti-mycobacterial agents

Table 1 lists the results obtained by the BACTEC, flow cytometric and proportion methods for 26 clinical isolates of *M. tuberculosis* with different susceptibilities to isoniazid, rifampicin and ethambutol. There was 94% agreement (73/78 tests) between the proportion and flow cytometric methods for isoniazid. Three of the five discrepancies (isolates 15, 16 and 23) occurred with isoniazid 0.2 mg/L. The remaining discrepancies occurred with isolates 19 and 20 at isoniazid 1 and 5 mg/L, respectively. These five isolates were classed as resistant to isoniazid by the proportion method, but as susceptible to isoniazid by the flow cytometric method. No discrepancies between the methods were detected when susceptibility to ethambutol or rifampicin was tested for the 26 isolates.

In contrast, there was considerable disagreement between the proportion and BACTEC methods regarding susceptibility to isoniazid, with 17 (22%) discrepancies among the 78 tests. Nine of the discrepancies occurred with isoniazid 0.2 mg/L. Isolates 1, 11, 14, 15, 16, 23 and 24 were resistant to isoniazid by the proportion method, but susceptible by the BACTEC method. The remaining isolates (5 and 12) were susceptible by the proportion method, but resistant by the

Table 1. Results of susceptibility tests for 26 isolates of *Mycobacterium tuberculosis* exposed to isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) by the proportion, flow cytometric and BACTEC methods

	Susceptibility to indicated concentrations of INH, RIF and EMB (mg/L)														
	Proportion method					Flow cytometric method					BACTEC method[a]				
	INH			RIF	EMB	INH			RIF	EMB	INH			RIF	EMB
Isolate	0.2	1	5	1	5	0.2	1	5	1	5	0.2	1	5	1	5
1	|R|	S	S	S	S	**R**	S	S	S	S	|**S**|	S	S	S	S
2	R	R	|S|	S	S	R	R	**S**	S	S	R	R	|**R**|	S	S
3	R	|S|	S	S	S	R	**S**	S	S	R	|**R**|	S	S	S
4	S	S	S	|R|	S	S	S	S	**R**	S	S	S	S	|**S**|	S
5	|S|	S	S	|R|	S	**S**	S	S	**R**	S	|**R**|	S	S	|**S**|	S
6	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S
7	R	R	|S|	S	S	R	R	**S**	S	S	R	R	|**R**|	S	S
8	R	R	R	S	S	R	R	R	S	S	R	R	R	S	S
9	R	R	R	S	R	R	R	R	S	R	R	R	R	S	R
10	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S
11	|R|	S	S	S	S	**R**	S	S	S	S	|**S**|	S	S	S	S
12	|S|	|S|	S	R	S	**S**	**S**	S	R	S	|**R**|	|**R**|	S	R	S
13	R	R	|S|	S	S	R	R	**S**	S	S	R	R	|**R**|	S	S
14	|R|	S	S	|S|	S	**R**	S	S	**S**	S	|**S**|	S	S	|**R**|	S
15	|R|[B]	S	S	S	S	S	S	S	S	S	|S|	S	S	S	S
16	|$\overline{\text{R}}$|	S	S	S	S	$\overline{\text{S}}$	S	S	S	S	|S|	S	S	S	S
17	$\overline{\text{R}}$	R	S	S	S	$\overline{\text{R}}$	R	S	S	S	R	R	S	S	S
18	R	R	|S|	S	S	R	R	**S**	S	S	R	R	|**R**|	S	S
19	R	$\underline{\text{R}}$	|S|	S	R	R	**S**	**S**	S	R	R	**R**	|**R**|	S	R
20	R	$\overline{\text{R}}$	$\underline{\text{R}}$	S	S	R	$\overline{\text{R}}$	**S**	S	S	R	R	**R**	S	S
21	R	|S|	$\overline{\text{S}}$	S	|S|	R	**S**	$\overline{\text{S}}$	S	**S**	R	|**R**|	S	S	|**R**|
22	R	R	R	S	R	R	R	R	S	R	R	R	R	S	R
23	|R|	S	S	R	S	S	S	S	R	S	|S|	S	S	R	S
24	|$\overline{\text{R}}$|	S	S	S	|R|	$\overline{\text{R}}$	S	S	S	**R**	|**S**|	S	S	S	|**S**|
25	S	S	S	|R|	S	S	S	S	**R**	S	S	S	S	|**S**|	S
26	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S

Bold type indicates discrepancies between the flow cytometric and BACTEC methods.
Underlining indicates discrepancies between the flow cytometric and proportion methods.
Vertical lines indicate discrepancies between the proportion and BACTEC methods.
[a]The INH concentrations used in the BACTEC method were 0.1, 0.4 and 2.5 mg/L, equivalent to 0.2, 1 and 5 mg/L used in the proportion [4] and flow cytometric methods. Likewise, EMB 2.5 mg/L and RIF 2 mg/L were used (see text).

BACTEC method. Furthermore, eight discrepancies were detected with isoniazid 1 and 5 mg/L, with isolates 2, 3, 7, 12, 13, 18, 19 and 21 susceptible by the proportion method, but resistant by the BACTEC method.

When susceptibility to ethambutol and rifampicin was tested by the proportion and BACTEC methods, an additional six discrepancies were found. Three isolates (4, 5 and 25) were resistant to rifampicin by the proportion method, but susceptible by the BACTEC method. The remaining isolate (14) was susceptible to rifampicin by the proportion method, but resistant by the BACTEC method. Furthermore, contrasting ethambutol susceptibility results were obtained for isolates 21 and 24 by these two methods.

The susceptibility results were also compared between the flow cytometric and BACTEC methods. Sixteen (20%) discrepancies were found when testing isonaizid susceptibility, while four (15%) and two (8%) discrepancies, respectively, were detected when testing rifampicin and ethambutol susceptibilities. These percentages were similar to those obtained when the BACTEC method was compared with the proportion method. Table 2 summarises the above results. No discrepancies were found among the test methods when the reference strains ATCC 27294, 35822, 35838 and 35837 were tested.

Analysis of discordant results

Five isolates (15, 16, 19, 20 and 23) accounted for all the isoniazid discrepancies between the proportion and the flow cytometric methods. Since the proportion method is considered to be the reference method [2,4], the flow cytometric susceptibility tests were repeated with these isolates, but identical results were obtained. When a broader range of isoniazid concentrations (0.06–5 mg/L) was tested by the flow cytometric assay (Table 3), four of the five isolates (15, 16, 19 and 23) were susceptible to a lower concentration of isoniazid than observed previously with the same method (0.12, 0.12, 0.5 and 0.06 mg/L, respectively). However, isolate 20 remained susceptible only to isoniazid 5 mg/L. Most likely, the resistant result obtained by the proportion method was correct.

Effects of inoculum size on flow cytometric results

Three isolates (1, 11 and 14) were selected because they were resistant to isoniazid ≤0.2 mg/L. Table 4 shows the susceptibility patterns of these isolates over a range of isoniazid concentrations from 0.06 to 5 mg/L. These isolates were resistant to isoniazid 0.2 mg/L with an inoculum of 10^6 CFU/mL, but were susceptible to 0.12 mg/L when the inoculum was reduced to 10^4 CFU/mL.

Table 2. Summary of discrepancies observed between susceptibility testing methods for isoniazid, rifampicin and ethambutol with 26 isolates of *Mycobacterium tuberculosis*

	Number of discrepancies observed with the indicated concentrations (mg/L)							
	Isoniazid				Rifampicin		Ethambutol	
Methods	0.2[a]	1	5	%	1	%	5	%
Proportion vs. flow cytometry	3	1	1	6	0		0	
Proportion vs. BACTEC	9	3	5	22	4	15	2	8
Flow cytometry vs. BACTEC	6	4	6	20	4	15	2	8

There were 26 tests/concentration of anti-mycobacterial agent.
[a]The isoniazid concentrations used in the BACTEC method were 0.1, 0.4 and 2.5 mg/L, equivalent to 0.2, 1 and 5 mg/L used in the proportion [4] and flow cytometric methods. Likewise, ethambutol 2.5 mg/L and rifampicin 2 mg/L were used (see text).

Table 3. MICs of isoniazid obtained by flow cytometry for discordant isolates

	Susceptibility to indicated concentrations of isoniazid (mg/L)						
Discordant isolates	0.06	0.12	0.2	0.5	1.0	2.5	5.0
15	R	S	S	S	S	S	S
16	R	S	S	S	S	S	S
19	R	R	R	S	S	S	S
20	R	R	R	R	R	R	S
23	S	S	S	S	S	S	S

R, resistant; S, susceptible.

Table 4. Effect of inoculum size on isoniazid susceptibility results obtained by flow cytometry

		Susceptibility to indicated concentration of isoniazid (mg/L)						
Isolate no.	Inoculum size	0.06	0.12	0.2	0.5	1.0	2.5	5.0
1	10^6	R	R	R	S	S	S	S
	10^4	R	S	S	S	S	S	S
11	10^6	R	R	R	S	S	S	S
	10^4	R	S	S	S	S	S	S
14	10^6	R	R	R	S	S	S	S
	10^4	R	S	S	S	S	S	S

R, resistant; S, susceptible.

DISCUSSION

The proportion method is considered to be the reference method for susceptibility testing of *M. tuberculosis* [2,4]. The method has proven useful in assisting clinicians to choose or confirm effective therapy for eradication of *M. tuberculosis* in patients. Resistance or susceptibility is determined by comparing the number of colonies that grow on a drug-containing plate with the number of colonies growing on the control medium. An isolate is considered resistant if ≥1% grow on the drug-containing medium. Results are generally available 3 weeks after inoculation of the plate with an isolate of *M. tuberculosis* [2]. As the proportion method was used as the reference standard in the present study, the BACTEC or flow cytometric methods could not be shown to be better than the proportion method.

Several methods have been developed that decrease greatly the time required by the proportion method to obtain susceptibility test results [11–20]. These methods include a bioluminescence assay for detection of mycobacterial ATP [11], the Gen-Probe DNA hybridisation system [12], the luciferase reporter gene assay [14], high-performance liquid chromatography mycolic acid analysis [15], the Etest method [16] and a colorimetric method [17]. However, to date, only the BACTEC method has been recognised as a valid alternative [7,8]. This method involves measurement of $^{14}CO_2$ produced by mycobacteria growing in broth containing ^{14}C-labelled palmitic acid with or without anti-mycobacterial agents. Most importantly, reproducible data are available 4–12 days after initiation of the procedure. The BACTEC is now accepted as a valid replacement for the proportion method [4].

The present study compared susceptibility results obtained by the proportion method with those obtained by the flow cytometric and BACTEC methods for 26 clinical isolates of *M. tuberculosis*. Overall, there was agreement between the proportion and flow cytometric methods for 73 (94%) of 78 isoniazid tests, and four of the five discrepancies were resolved with an increase in isoniazid concentration. Complete agreement was found for ethambutol and rifampicin. In contrast, the proportion and BACTEC methods failed to agree on 17 (22%) of the 78 isoniazid tests, and similar findings were observed when the flow cytometric and BACTEC methods were compared. Additional discrepancies were detected for rifampicin (15%) and ethambutol (8%).

The study was based on the assumption that the susceptibility results obtained by the proportion method were correct, but selection of a subpopulation of resistant or susceptible organisms within the cell population being tested could have affected the results obtained. However, this seems unreasonable on the basis of the present data, because the overall agreement between the proportion and flow cytometric methods was 94%. Moreover, if the low concentrations (0.2 and 1 mg/L) of isoniazid were eliminated from the analysis, agreement between the methods approached 100%. A more likely explanation for the minor discrepancies detected with isoniazid 0.2 and 1 mg/L by the proportion method is decay of isoniazid. It is known that the bactericidal activity of isoniazid decreases rapidly in medium after incubation for 7 days [24,25], but little decay of isoniazid to isonicotinic acid and other metabolites would be expected in the 24-h period required to obtain susceptibility results by the flow cytometric method. In support of this hypothesis, the resistant (0.2 mg/L and 1 mg/L) isolates detected by the proportion method were susceptible to even lower concentrations of isoniazid when retested by the flow cytometric method. Moreover, it was shown that the inoculum size can affect the flow cytometric results, with increased susceptibility to isoniazid being observed when the inoculum was decreased from 10^6 to 10^4 CFU. This would account for four of the five isoniazid discrepancies found between the flow cytometric and proportion methods.

The isoniazid susceptibility results obtained by the BACTEC method were disappointing when compared to those obtained by the proportion method. An explanation for the 17 discrepancies is difficult to find, especially when the proportion and flow cytometric methods had 94% agreement. The BACTEC results cannot, however, be attributed to faulty quality control, since only minor variations occurred on retesting, and the reference strains (ATCC 27294, 35822, 35838 and 35837) were within limits. It is possible that other laboratories would obtain similar results if the proportion method was run concurrently with the BACTEC method. In addition, laboratory personnel may

require continuous training to maintain quality with the BACTEC procedure. These steps are not normally undertaken, but should be considered in order to improve the quality of results.

Safety is a major concern when performing susceptibility testing by flow cytometry for *M. tuberculosis*. Most laboratories will be reluctant to use or evaluate the method because of perceptions that an infectious aerosol may be generated. Although this is possible, the danger can be prevented by killing the mycobacteria before testing. In the present study, all data were obtained after treating samples with paraformaldehyde 1% v/v, and it has been shown that viable *M. tuberculosis* cells are not recovered following treatment with paraformaldehyde for 40 min [20]. Increasing the period of exposure to paraformaldehyde to 24 h did not alter the results obtained by flow cytometry. However, viable *M. tuberculosis* cells may still be trapped in the lips or caps of the paraformadehyde-treated susceptibility tubes. Therefore, samples should still be processed in a Biosafety Level 3 tuberculosis facility. Since few clinical and reference mycobacterial laboratories have a flow cytometer available in or near the laboratory, susceptibility testing by flow cytometry will be limited. This problem can be overcome by transferring the paraformaldehyde-treated samples to a new vial containing paraformaldehyde, whereupon the samples are safe for processing outside the mycobacterial laboratory. Nevertheless, expertise with flow cytometry is absolutely necessary to ensure safety and the quality of the data. The test is rapid and does not require multiplication of *M. tuberculosis*. However, a cooperative study involving several laboratories and fresh clinical isolates of *M. tuberculosis* is required to confirm the present results.

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